BD1 CIP FWC II

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Sherie L. Morrison, et al.
Serial No.	:	07/675,106
Filed	:	March 25, 1991
For	:	RECEPTORS BY DNA SPLICING AND EXPRESSION
Group	:	185
Examiner	:	T. Nisbet

#22

Hon. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

DECLARATION OF VERNON T. OI
PURSUANT TO 37 C.F.R. § 1.131

I, VERNON T. OI, declare that:

1. I am a co-inventor in the above-identified patent application.

2. I am aware of the following publication by Ochi et al., "Functional Immunoglobulin M Production After Transfection Of Cloned Immunoglobulin Heavy And Light Chain Genes Into Lymphoid Cells", Proc. Nat'l Acad. Sci. USA, vol. 80, pp. 6351-55 (Oct. 1983), and am informed and believe that it was mailed to subscribers of the journal in which it was published on October 19, 1983. A copy of that article is attached hereto as Exhibit A.

3. I make this declaration to establish, when read in conjunction with the declaration of Sherie L. Morrison, one of my co-inventors, the conception of a functional antibody produced by a mammalian cell prior to the October 19, 1983 effective date of the Ochi reference and to establish the diligent reduction to practice of the invention from a time prior to said date.

4. Conception of the invention is evidenced by the grant application that I submitted to Becton Dickinson with Leonard A. Herzenberg and by the grant application that Sherie L. Morrison submitted to the American Cancer Society. The dates of those applications are prior to October 19, 1983. Copies of the pertinent portions of the applications are attached as Exhibit B and Exhibit C respectively.

5. Prior to October 19, 1983, I completed construction of a vector (designated HuK) containing a gene coding for the light chain of an antibody. Evidence of this work is contained in the true copies of pages from my laboratory notebook attached hereto as Exhibit D. Those pages are dated prior to October 19, 1983. I also completed construction of a vector (designated HuG) containing a gene coding for the heavy chain of an antibody. Evidence of this work is contained in the true copies of pages from the laboratory notebook of Tim Gadus, a laboratory technician with whom I worked in the laboratory of Leonard Herzenberg, attached hereto as Exhibit E. Those pages are dated prior to October 19, 1983. These vectors contained regulatory sequences making them suitable for transfection into and expression in mammalian cell lines.

6. From a time prior to October 19, 1983 until November 23, 1983, I was occupied with tasks related to changing jobs. I was preparing to leave the laboratory where I was working at Stanford University in order to accept a position as Senior Scientist at Becton Dickinson. As set forth in my offer letter (a true copy of which is attached hereto as Exhibit F) which is dated prior to October 19, 1983, I was to be in charge of a new laboratory that would be constructed at Becton Dickinson. It was further anticipated that I would hire employees to staff the

laboratory and would administer a $200,000 capital expense budget.

7. I began work at Becton Dickinson on December 1, 1983 as shown by the true copy of the Personnel Change Notice attached hereto as Exhibit G. Prior to December 1, 1983, and during the time period after the October 13, 1983 mailing of the Ochi reference and prior to the shipment of the vectors to Dr. Morrison on November 23, 1983, I was fully involved in making preparations and performing administrative tasks related to the laboratory that I was to run at Becton Dickinson. In addition to purchasing laboratory equipment, I was involved in the hiring process for my research staff. Eventually four people were hired as a result of this process. In particular, Ginger Bryan Waters was offered a position on December 19, 1983 to begin work in my laboratory as evidenced by the copy of her offer letter appended hereto as Exhibit H.

8. On November 23, 1983 the HuK and HuG vectors that I had constructed were sent to Dr. Morrison at my request from Dr. Herzenberg's laboratory by Tim Gadus. These materials were typically sent via the United States Postal Service, regular mail. In this case, November 24, 1983 was Thanksgiving Day. Therefore, Dr. Morrison likely did not receive these materials until the following Monday, November 28, 1983. A copy of the transmittal record evidencing this shipment is attached hereto as Exhibit I.

9. I further declare that all statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment or both under

Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application or any patent issuing thereon.

By _____
Vernon T. Oi

Dated: January 3, 1992

#22½

BD1 CIP FWC II

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Sherie L. Morrison, et al.
Serial No.	:	07/675,106
Filed	:	March 25, 1991
For	:	RECEPTORS BY DNA SPLICING AND EXPRESSION
Group	:	185
Examiner	:	T. Nisbet

Hon. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

DECLARATION OF SHERIE L. MORRISON
PURSUANT TO 37 C.F.R. § 1.131

I, SHERIE L. MORRISON, declare that:

1. I am a co-inventor in the above-identified patent application.

2. I am aware of the following publication by Oohi et al., "Functional Immunoglobulin M Production After Transfection Of Cloned Immunoglobulin Heavy And Light Chain Genes Into Lymphoid Cells", Proc. Nat'l Acad. Sci. USA, vol. 80, pp. 6351-55 (Oct. 1983), and am informed and believe that it was mailed to subscribers of the journal in which it was published on October 19, 1983. A copy of that article is attached hereto as Exhibit A.

3. I make this declaration to establish, when read in conjunction with the declaration of Vernon T. Oi, one of my co-inventors, conception of a functional antibody produced by a mammalian cell prior to the October 19, 1983 effective date of the Ochi reference and to establish diligent reduction to practice of the invention from a time prior to said date. The conception and reduction to practice of a functional antibody is evidenced by the true copies of pages from my laboratory notebook and from the laboratory notebook of my staff research associate, Latitia Wims, who worked closely with me and under my direction during the reduction to practice of this invention, and from other laboratory records attached hereto.

4. Conception of the invention is evidenced by the grant application that I submitted to the American Cancer Society and by the grant application that Leonard A. Herzenberg and Vernon T. Oi submitted to Becton Dickinson. The dates of those applications are prior to October 19, 1983. Copies of the pertinent portions of the applications are attached as Exhibit B and Exhibit C respectively.

5. Construction of vectors containing genes coding for the light and heavy chains of an antibody (designated HuK and HuG respectively) was completed by Dr. Vernon T. Oi prior to October 19, 1983. (See Declaration of Vernon T. Oi, ¶ 5.) These vectors contained regulatory

sequences making them suitable for transfection into and expression in mammalian cell lines.

6. Between November 25, 1983 and November 29, 1983 I received the HuK and HuG vectors that had been constructed by Dr. Oi. On November 29, 1983, I co-transfected J558L myeloma cells with the HuK and HuG vectors. True copies of my notebook pages evidencing this work are attached as Exhibit D.

7. When I performed the transfections recited in the preceding paragraph, I followed our standard laboratory protocol for protoplast fusion. A copy of this standard protocol is attached as Exhibit E. Typically, work done before "Fusion Day" is not recorded in a lab notebook as it is routine and merely preparatory in nature. Similarly, the addition of nutrients or selective media in the days following the fusion are not recorded either.

8. Following our standard protocol, the cell culture typically requires six weeks of growth before it contains a sufficient number of cells for detailed analysis including radio-labelling and cryopreservation.

9. On January 9, 1984, six weeks after the transfections, my staff research associate, Letitia Wims, labelled 24 cell lines that resulted from the fusions that I performed on November 29. These cell lines are denoted TAO.

True copies of the page of Ms. Wims' laboratory notebook that documents this work are attached as Exhibit F.

10. On January 10, 1984, Ms. Wims performed SDS gel electrophoresis on the expression product of the TAO cell line. The result of that procedure showed that the expression product contained proteins that had the molecular weight of the expected immunoglobulin proteins. True copies of Ms. Wims' laboratory notebook pages documenting this procedure are attached as Exhibit G.

11. We subsequently performed experiments designed to verify that the antibodies that had been produced and isolated on January 10, 1984 were functional, i.e. capable of specific antibody binding. Initially, we ran a series of ELISA assays. On January 17 and 18, 1984, we performed ELISA assays of the TAO cell line supernatants using plates coated with PC-KLH and attempted to detect antigen binding with ^{125}I-Protein A. Neither trial successfully demonstrated binding. I believe that the difficulty that we had demonstrating binding was due, at least in part, to the quality of the ^{125}I-Protein A which was old. True copies of Ms. Wims' laboratory notebook pages documenting this work are attached as Exhibit H.

12. We then repeated the ELISA assay using fresh ^{125}I-Protein A received on January 27, 1984. On January 30, 1984 we standardized the Protein-A binding assay and

determined that it worked. Immediately thereafter, on January 30, 1984, we again tested the TAO supernatants by ELISA. One well (TAO-18) tested positive, another somewhat positive and two others slightly positive for antigen binding. True copies of pages from the laboratory notebook of Ms. Wims documenting this work are attached as Exhibit I.

13. Our next analysis of antigen binding was performed by running biosynthetically-labeled culture supernates and cell lysates from the TAO cell line through a PC column equilibrated with PBS. An overnight label of the TAO-18 cells was begun on February 2, 1984. On February 6, 1984, we ran the labelled supernatant and lysate through the column, but did not detect binding. True copies of pages from the laboratory notebook of Ms. Wims documenting this work are attached as Exhibit J.

14. Additional ELISA assays for PC binding were run in February of 1984. On February 15, 1984, an ELISA again showed binding of antigen by the original supernatant from TAO-18. However, a new preparation failed to show binding activity. We became concerned that TAO was changing and losing the ability to produce the transfected light chain gene. A true copy of the page from the laboratory notebook of Ms. Wims documenting this work is attached as Exhibit K.

15. To address this problem, we attempted to increase light chain production by TAO-18 by transiently transfecting it with the HuK vector and then harvesting the cells after 96 hours of growth to allow light chain expression. TAO was transfected on February 17, 1984, and labeled with ^{35}Met on February 21, 1984. I put the labeled supernatant through the PC column on February 22, 1984. We precipitated the product eluted from the column with Staph A and ran it on an SDS gel on March 5, 1984. The results suggested specific binding, but the product was suboptimal. True copies of pages from the laboratory notebook of Ms. Wims and of the gel are attached as Exhibit L.

16. The TAO cell line was the result of a transfection of the J558L cell line, a cell line which produces endogenous lambda light chain. Because of our concern that the lambda chain was interfering with binding assays, on February 8, 1984 we attempted transfection of cell lines which produced no immunoglobulin chains. On March 28, 1984, an RIA was run on such new transfects, but none were positive for antigen binding. True copies of pages from my laboratory notebook and that of my research assistant, Letitia Wims, documenting this work are attached as Exhibit M.

SLM 1/3/92

17. In the original vectors obtained from Vernon T. Oi, the selectable marker for both the heavy and the

light chain was gpt. Therefore, there was no way to ascertain if the cells had indeed taken up both vectors. To address that concern, I decided to change the selectable marker on the light chain vector to neo. Digestions were done on February 21, 1984, and March 11, 1984, to produce fragments for clonings; however, both efforts were unsuccessful. A third attempt on March 24, 1984, was successful, yielding the vectors neo-HuK1 and neo-HuK3. These were used to transfect TAO-6 and TAO-18 on March 28, 1984. Clone 27 from TAO-6 was later analysed in May (See ¶ 18). True copies of the pages from my laboratory notebook documenting this work are attached as Exhibit N.

18. The February 15, 1984, ELISA (discussed previously in paragraph 14 of this declaration) did not show positive results for other supernatants from newer colonies of the TAO cell line suggesting that the cell line had mutated and lost the ability to produce one of the chains of the antibody. Transient expression data (See ¶ 15) suggested it was the light chain that was lost. One potential problem was the selectable markers on the vectors. We therefore attempted to recover production of the antibody by retransfecting the cells with neo-HuK (See ¶17). On March 28, 1984, I made protoplasts of Neo-HuK TAO-6 and fused them with Tao-6 and Tao-18 neo-HuK to augment light chain production. On March 30, 1984, I added selective medium to the transfectants.

SLM 1/3/92

The colonies were then grown up for the standard six-week period until May 2, 1984. On May 2, 1984, I ran the supernatant from a colony of the newly-transfected TAO-6 through a PC column and made three pools; the pool with fractions 2-8 bound Staph A. At this time, I also realized that the phosphate buffer I had been using for the column was inappropriate. On May 4, 1984, I labeled the TAO-6 supernatant overnight with ^{14}C-VTL and then ran it through a PC column. The results of this analysis indicated specific antigen binding. The material eluting from the column was bound by Staph A and by monoclonal antibodies specific for the idiotype of the original murine antibody. True copies of pages from my laboratory notebook and Ms. Wins' laboratory notebook documenting this work are attached as Exhibit O.

SLM 1/3/92

19. I further declare that all statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment or both under

Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application or any patent issuing thereon.

By Sheria L. Morrison
Sheria L. Morrison

Dated: January 3, 1992

Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 6351–6355, October 1983
Immunology

Functional immunoglobulin M production after transfection of cloned immunoglobulin heavy and light chain genes into lymphoid cells

(protoplast fusion/G418 selection)

ATSUO OCHI*†, ROBERT G. HAWLEY*†, TERESA HAWLEY*†, MARC J. SHULMAN†‡, ANDRÉ TRAUNECKER§, GEORGES KÖHLER§, AND NOBUMICHI HOZUMI*†

*Ontario Cancer Institute and †Department of Medical Biophysics, University of Toronto, 500 Sherbourne Street, Toronto, ON M4X 1K9 Canada; ‡Rheumatic Disease Unit, Wellesley Hospital, Toronto, ON M4Y 1J3 Canada, and §Basel Institute for Immunology, Grenzacherstrasse 487, Basel CH-4005, Switzerland

Communicated by Niels Kaj Jerne, July 11, 1983

ABSTRACT **The rearranged immunoglobulin heavy (μ) and light (κ) chain genes cloned from the Sp6 hybridoma cell line producing immunoglobulin M specific for the hapten 2,4,6-trinitrophenyl were inserted into the transfer vector pSV2-neo and introduced into various plasmacytoma and hybridoma cell lines. The transfer of the μ and κ genes resulted in the production of pentameric, hapten-specific, functional IgM.**

Work over the last decades has provided extensive information on immunoglobulin function and structure (1). Despite this information, it has been possible only in gross terms to relate molecular function with particular structural features.

With the advent of genetic engineering and gene transfer techniques, questions regarding structure–function relationships can now be readily addressed—that is, virtually any gene segment can be modified precisely *in vitro* and the novel segment can then be exchanged with its normal counterpart. By introducing such engineered genes into the appropriate cells, the effects of systematic alterations in protein structure on protein function can be assessed.

Because immunoglobulin production is a specialized function of cells of the B-lymphocyte lineage, it is expected that the conditions for proper Ig gene expression will be provided only in appropriate immunocompetent cells. For example, to produce normal pentameric IgM(κ), a cell must transcribe, process, and translate RNA for the μ and κ chains and also provide J protein, enzymes for the proper polymerization and glycosylation of the Ig chains, as well as a suitable secretory apparatus. We have previously described a system for transferring a functional immunoglobulin κ light chain gene into IgM-producing hybridoma cells (2). Here we extend this work to show that the transfer of the μ and κ chain genes of a defined specificity into various plasmacytoma and hybridoma cell lines results in the production of functional pentameric, hapten-specific IgM(κ).

MATERIALS AND METHODS

Cell Lines. X63Ag8 was originally derived (3) from the plasmacytoma MOPC21 and synthesizes IgG1(κ) of unknown specificity. X63Ag8.653 was derived from X63Ag8 as a subclone that synthesizes neither the heavy (γ1) nor light (κ) chain (4). Similarly, Sp2/0Ag14 is an Ig nonproducing subclone of the Sp2 hybridoma (5). Sp6 is a hybridoma making IgM(κ) specific for the hapten 2,4,6-trinitrophenyl (TNP); originally this cell line produced the γ1 and κ chains of X63Ag8 as well as the (TNP specific) μ_{TNP} and κ_{TNP} chains (6). A subclone of Sp6 not making the γ1 chain was isolated, and the Sp602 and Sp603 cell lines were derived from this γ1 nonproducer. The mutant cell line igm-10, derived from Sp602 (7), lacks the gene encoding μ_{TNP} (8).

Gene Transfer. The construction of pSV2-neo plasmid vectors carrying the genes for μ_{TNP} or κ_{TNP} or both is described in the text. The vectors were transfected into the $r_k^- m_k^-$ *Escherichia coli* strain K803. To transfer the vector, bacteria bearing the appropriate plasmids were converted to protoplasts and fused to the indicated cell lines as described (2). The frequency of G418-resistant transformants per input cell was approximately 10^{-4} for X63Ag8 and Sp2/0Ag14, 10^{-5} for igm-10, and 10^{-6} for X63Ag8.653.

Analysis of Ig. As described previously (7), Ig was biosynthetically labeled, in the presence or absence of tunicamycin, immunoprecipitated, and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis with or without disulfide bond reduction. TNP binding IgM was assayed by TNP-dependent hemagglutination and by TNP-dependent enzyme-linked immunoadsorbent assay (ELISA) as described (2, 7). The hemolyses of protein A-coupled erythrocytes and TNP-coupled erythrocytes were used to assay total IgM- and TNP-specific complement activating IgM, respectively (7).

Analysis of RNA and DNA. Cytoplasmic RNA was isolated according to Schibler *et al.* (9) and subjected to RNA blot analysis as described by Thomas (10).

Procedures for DNA extraction (11), nitrocellulose blotting (12), and radiolabeling of probes (13) have been described (14, 15). Probes specific for genes encoding immunoglobulin constant and variable regions are detailed in the figure legends.

RESULTS

Description of Vectors and Expression Systems. The hybridoma cell line Sp6 secretes IgM(κ) specific for the hapten TNP. We have previously described the cloning of the TNP-specific κ gene, designated Tκ1 (16), and the construction of the recombinant, pR-Tκ1, where Tκ1 is inserted in the *Bam*HI site of the vector pSV2-neo (2, 17). The μ_{TNP} gene was cloned in λCh4A from *Eco*RI partially digested DNA of Sp6 cells, and this clone is designated Sp6-718. The 16-kilobase-pair (kbp) fragment carrying the variable and constant regions was obtained from Sp6-718 after partial digestion with *Eco*RI and was inserted at the *Eco*RI site of the vectors pSV2-neo and pR-Tκ1. In these recombinants, designated pR-Sp6 and pR-HL_{TNP}, re-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: TNP, 2,4,6-trinitrophenyl; ELISA, enzyme-linked immunoadsorbent assay; kbp, kilobase pair(s); SV40, simian virus 40; kb, kilobase(s).